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Bacterial active efflux pumps: a lifestyle

For surviving in hostile conditions, or for conquering new territories, bacteria need to have some control over their immediate environment. In particular, most possess the capacity to reject foreign molecules that would have produced serious damage by intracellular penetration (antibiotics and biocides, for example). In addition, pumps can extrude poorly diffusible or toxic endogenous molecules from the cytosol. These pumps, or active efflux systems, are substrate transporters requiring energy generated by adenosine triphosphate (ATP) or proton motive pumps for their function. Several dozen bacterial pumps have been described so far, belonging to five superfamilies as described in Figure 1 and Table 1. The major facilitation and ATP-binding cassette superfamilies cross the prokaryote/eukaryote borders.

Efflux pumps

The pump is a protein inserted into the cytoplasmic membrane, often composed of 12 transmembrane domains (Figure 2, page 3). It has been proposed that the tertiary structure includes two similar channels, each made from six transmembrane domains, allowing bi-directional crossing: one proton getting in, one substrate molecule getting out (anti-port). In Gram-negative bacteria, which possess two membranes, the pump molecule has to contain a porin structure in the outer membrane and a fusion periplasmic protein joining the two membranes (Figure 3, page 3).

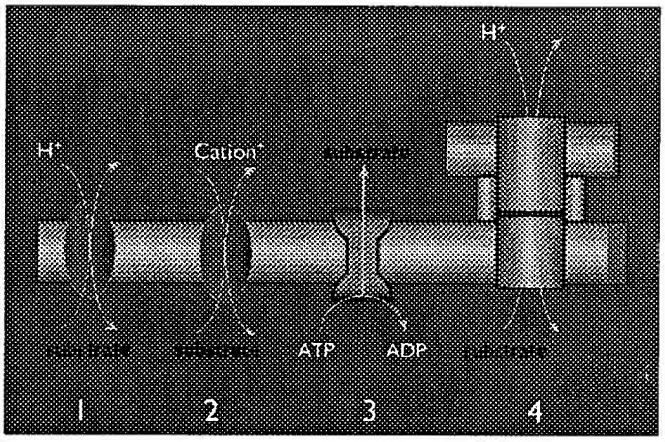

Figure 1: Classes of active efflux systems in bacteria: major facilitation superfamily (MFS) or small multidrug resistance (SMR)[1]; multiple antimicrobial extrusion (MATE)[2]; ATP binding cassette (ABC)[3]; resistance nodulation division (RND)[4].

Table I: Examples of active efflux systems, classified into superfamilies

Efflux superfamily	Typical example (bacterial species)	Antibiotic substrate profile
Major facilitation superfamily (MFS)	NorA (*Staphylococcus aureus*)	Quinolones, chloramphenicol
ATP binding cassette (ABC)	MrsA (*S. aureus*)	Macrolides
Small multidrug resistance (SMR)	EmrE (*Escherichia coli*)	Tetracycline
Multiple antimicrobial extrusion (MATE)	YdhE (*E. coli*)	Quinolones, trimethoprim
Resistance nodulation division (RND)	Multidrug efflux transporter B (MexB)	Most β-lactams, quinolones, tetracycline, macrolides, chloramphenicol, rifampin, trimethoprim, sulfonamides, fusidic acid

ATP, Adenosine triphosphate.

The function of efflux systems

Active efflux systems can serve several functions. In *Pseudomonas aeruginosa*, efflux systems have been associated with virulence, since efflux deficient mutants exhibit diminished capacity for invasion in experimental models.[1] Multidrug efflux transporter F (MexF) in *P. aeruginosa* contributes to quorum-sensing regulation affecting secretion of virulence factors.[2] Export of toxins or locally produced antibiotics are also possible functions.[3,4] A very

continued on page 3

continued from page 1

important function is intrinsic and acquired antibiotic resistance. Most antibiotic classes can be affected by efflux, as shown in Table 1. In Gram-negative bacteria, activation or repression of efflux systems, such as those belonging to the resistance nodulation division (RND) family, can produce troublesome multi-resistant phenotypes. Of particular concern is the fact that one substrate can select resistance to structurally unrelated antibiotics. Quinolones for example can select for carbapenem resistance.[5] Since some efflux include biocides in their substrate spectrum,[6] biocides can induce antibiotic resistance, at least *in vitro*, but the molecular basis of such large substrate profiles is not fully understood. It has been proposed that the pump may recognize amphophilic molecules, the hydrophobic moiety being inserted into the cytoplasmic membrane. The recent recognition, however, that aminoglycosides could be efflux substrates does not fit with this view.[7] Within a given antibiotic class, all molecules are not similarly affected by efflux resistance. For example, PmrA in *Streptococcus pneumoniae* extrudes ciprofloxacin about eight times more efficiently than moxifloxacin or gatifloxacin. In *P. aeruginosa*, meropenem but not imipenem is a substrate of the MexB pump. In streptococci, macrolide resistance by efflux is limited to 14- and 15-membered compounds. Efflux mechanisms in general, confer a low to moderate level of resistance (1- to 64-fold increase in minimal inhibitory concentration),[8] which is not necessarily clinically significant, particularly in Gram-positive organisms. Efflux resistance is also often associated with other resistance mechanisms, such as topoisomerase mutations in the case of quinolones, or outer membrane permeability alterations with the carbapenems. Efflux activation may allow delayed entry of antibiotic molecules, providing more time for selection of a second resistance mechanism. Many efflux genes are located on the bacterial chromosome, but others, such as Tet transporters, are found on plasmids.

Future developments

Active ongoing research is trying to develop anti-efflux strategies that will allow circumvention of efflux resistance and restoration of wild-type susceptibility, a strategy already used successfully with β-lactamase inhibitors. A number of compounds, for example reserpine, omeprazole or phenothiazines, exert their inhibitory properties against both prokaryotic and eukaryotic efflux systems, limiting their clinical potential as pump inhibitors. More specific compounds are, however, under investigation,[9] including tetracycline derivatives directed against Tet transporters, flavonolignans (natural alkaloids) active against NorA in *Staphylococcus aureus*, and an exciting series of small peptide inhibitors of RND transporters in *P. aeruginosa*.[10] ⁂

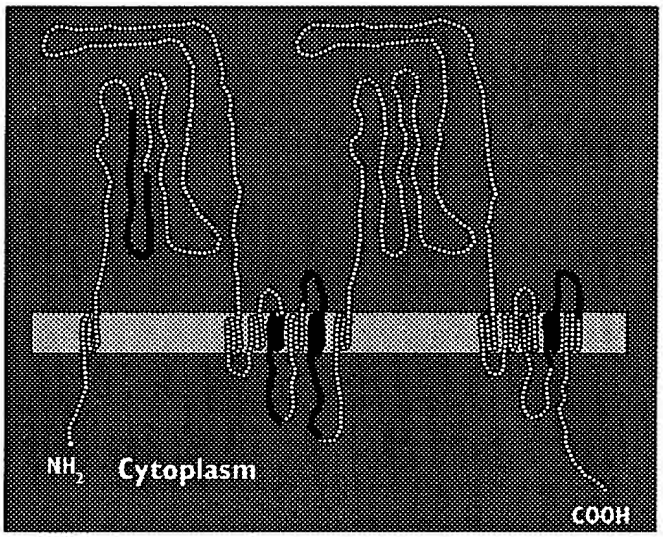

Figure 2: Two-dimensional model of a resistance nodulation division pump, with 12 transmembrane domains. This is thought to form two channels, one for the entry of a proton providing the energy, and the other for extrusion of the substrate molecule.[11]

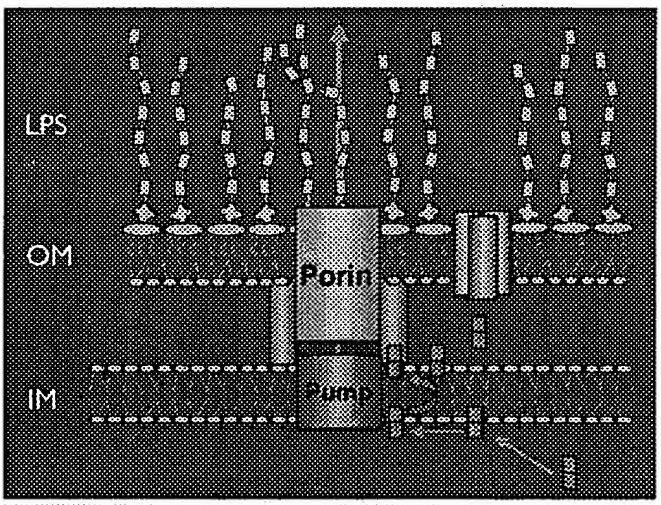

Figure 3: Model of the resistance nodulation division pump. Substrate amphophilic molecules (hydrophobic moiety in green), coming either from the outside or from the cytosol, are extracted from the cytoplasmic membrane by the pump.[12]

Jean-Claude Pechère
Universities of Geneva and Marrakech
President of the ISC

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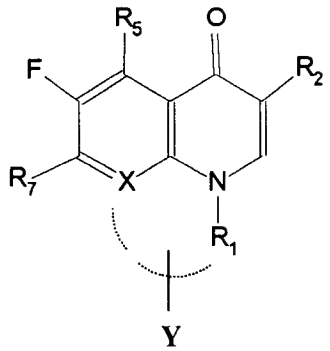
Table 1

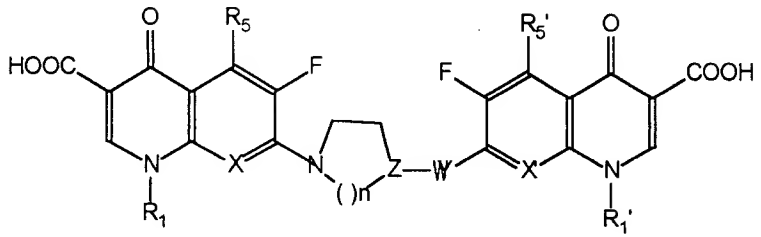
Some Preferred Compounds of the Invention Displaying Inhibition of the Efflux Pump of *Staphylococcus aureus* 1199 B (Nor A $^{+}$)



Entry	X	R_1	R_2	R_5	R_7	FIC INDEX #
1.	C-F	C_2H_5	COOH	H	4-acetoxypiperidin-1-yl	0.314
2.	C-F	C_2H_5	COOH	H	4-{2-(2-oxazolidin-1yl)ethyl}piperazin-1-yl	0.188
3.	C-H	c-C_3H_5	COOH	H	3,5-dimethyl-4-ethylpiperazin-1-yl	0.314
4.	C-H	c-C_3H_5	COOH	H	4-acetoxypiperidin-1-yl	0.282
5.	C-F	c-C_3H_5	COOH	NH2	(1,2,2,6,6-pentamethylpiperidin-4-yl)-N-methylamino	0.314
6.	N	c-C_3H_5	COOH	H	Pyrrolidin-3-ylamino	0.188
7.	C-F	$CH(CH_3)$-$CH_2CH_2SC_6H_5$	$COOC_2H_5$	H	4-hydroxypiperidin-1-yl	0.100
8.	C-H	$C_6H_4CF_3$(2)	COOH	H	3,3,4-trimethylpiperazin-1-yl	0.282
9.	C-F	$C_6H_4CF_3$(2)	COOH	NH2	morpholin-1-yl	0.314
10.	C-F	$C_6H_4CF_3$(2)	COOH	NH2	3,5-dimethylpiperazin-1-yl	0.314
11.	C-H	C_6H_4F(4)	COOH	H	4-(ethylamino)piperidin-1-yl	0.189
12.	C-F	$C_6H_3F_2$(2,4)	COOH	NH_2	3-hydroxy-5-methylpyrrolidin-1-yl	0.314
13.	C-F	$C_6H_3F_2$(2,4)	COOH	NH_2	3,3-dimethylpiperazin-1-yl	0.375
14.	N	$C_6H_3F_2$(2,4)	COOH	H	4-aminopiperidin-1-yl	0.314
15.	N	$C_6H_3F_2$(2,4)	$COOC_2H_5$	H	{(1α,5α,6α)-3-N-benzyl-3-azabicyclo[3.1.0]hex-6-yl} amino	0.185

16.	C-CH_2CH_2C**$H(CH_3)$	$COOCH_2COOC_2H_5$	H	4-hydroxy-piperidin-1-yl	0.400
17.	C-CH_2CH_2C*$H(CH_3)$	COO-piperidin-N-CH_3	H	4-hydroxy-piperidin-1-yl	0.370
18.	C-CH_2CH_2C*$H(CH_3)$	COO-CH_2CH_2-morpholine	H	4-hydroxy-piperidin-1-yl	0.180

Examples of Bis compounds of the invention Displaying Inhibition of the Efflux Pump of *Staphylococcus aureus* 1199 B (Nor A $^+$) (continuation of the table 1)



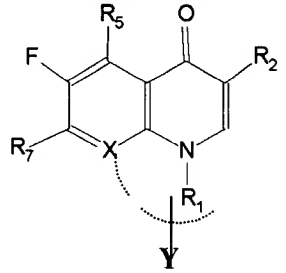
Entry	R_1	R_5	X	n	Z	Y	R_1'	R_5'	X'	FIC INDEX#
19	c-C_3H_5	H	CH	1	CH	NH	$C_6H_3F_2$(2,4)	H	N	0.253
20	c-C_3H_5	CH_3	CH	1	CH	NH	$C_6H_3F_2$(2,4)	H	N	0.320

- # MIC against *S. aureus* 1199B for all compounds is less than or equal to 6.25 μg/ml, except for entry Nos. 3 and 6, MIC is 3.12 μg/ml; for entry Nos. 5, 9, 18 MIC is 1.56 μg/ml. and for entry No 17 MIC is 0.8 μg/ml.

- ** R-isomer; *S-isomer; absence of an asterisk indicates that the substance is a racemic compound.

Table 2

Some Preferred Compounds of the Invention Displaying Inhibition of the Efflux Pumps of *Pseudomonas aeruginosa* 23587



Entry.	X	R_1	R_2	R_5	R_7	Difference in Zone diameter Z^1 (mm) #
1.	C-H	c-C_3H_5	COOH	CH_3	4-methoxypiperidin-1-yl	13
2.	C-OCH_3	c-C_3H_5	COOH	H	4-amino-3,3-dimethyl-piperidin-1-yl	12
3.	C-OCH_3	c-C_3H_5	COOH	H	4-hydroxy-3,3-dimethyl-piperidin-1-yl	8
4.	C-H	c-C_3H_5	COOH	H	3,5-dimethyl-4-ethylpiperazin-1-yl	8
5.	C-F	C_2H_5	COOH	H	(1∝,5∝,6∝)-6-amino-3-azabicyclo[3.1.0]hex-3-yl	7
6.	N	$C_6H_3(2,4\text{-}F_2)$	COOH	H	piperidin-4-ylamino	6
7.	N	c-C_3H_5	COOH	H	4-amino-3-ethyl-piperidin-1-yl	6
8.	C-CH_2CH_2 $CH(CH_3)$		COOH	H	4-(D-phenylalanyloxy)piperidin-1-yl hydrochloride	12
9.	C-$CH_2CH_2C^*H(CH_3)$		COOH	H	4-(D-leucyloxy)piperidin-1-yl hydrochloride	12
10.	C-$CH_2CH_2C^*H(CH_3)$		COOH	H	4-(L-alanyloxy)piperidin-1-yl hydrochloride	9
11.	C-CH_2CH_2 $C^{**}H(CH_3)$		COPhe-Lys-OMe	H	F	8
12.	C-CH_2CH_2 $CH(CH_3)$		COOH	H	trans-4-hydroxy-3-methyl-piperidin-1-yl	6
13.	C-$OCH_2CH(CH_3)$		COOH	H	4-hydroxy-3-ethyl-piperidin-1-yl	5

[1]Z = the difference of the diameter of the zone of inhibition in the levofloxacin containing plate minus the diameter of the zone of inhibition in the water containing control plate.

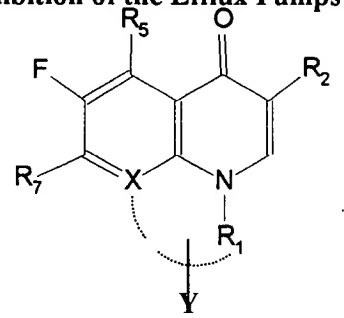
- # In the assay plates used, an agar well has a diameter of 7 mm; the minimum zone of inhibition observable is 8 mm in diameter. A zone less than 8 mm is considered as "nil".

The diameters of the zones of inhibition of the test substance listed in the water-containing plate was nil : 3, 8-9 mm : 9, 11mm : 1.

- ** R-isomer; * S-isomer; absence of an asterisk indicates that the substance is a racemic compound.

Table 3

Some Preferred Compounds of the Invention Displaying Inhibition of the Efflux Pumps of *Escherischia coli* 2051



Entry.	X	R_1	R_2	R_5	R_7	Difference in Zone diameter Z^1 (mm) #
1.	CH	c-C_3H_5	COOH	CH_3	Br	9
2.	C-OCH_3	c-C_3H_5	COOH	H	4-amino-3-methyl-piperidin-1-yl	10
3.	C-CH_2CH_2C*H(CH_3)		COOH	H	4-hydroxypiperidin-1-yl (0.2 H_2O)	9
4.	C-CH_2CH_2C*H(CH_3)		COOH	H	4-hydroxypiperidin-1-yl.[$(CH_3)_3N(OH)(CH_2)_2OH$] salt	9
5.	C-CH_2CH_2C*H(CH_3)		COOH	H	4-hydroxypiperidin-1-yl.1-Hydroxyethylpyrrolidine salt	11
6.	C-CH_2CH_2C*H(CH_3)		COOH	H	4-hydroxypiperidin-1-yl . Diethanolamine salt	11
7.	C-CH_2CH_2C*H(CH_3)		COOH	H	4-(D-leucyloxy)piperidin-1-yl hydrochloride	18
8.	C-CH_2CH_2C*H(CH_3)		$COOCH_2CH_2$ -morpholine	H	4-Hydroxypiperidin-1-yl	8
9.	C-CH_2CH_2C**H(CH_3)		CO-phe-lysOMe	H	F	11
10.	C-CH_2CH_2C*H(CH_3)		COOH	H	*trans*-3-methyl-4-hydroxypiperidin-1-yl	8

[1]Z = the difference of the diameter of the zone of inhibition in the levofloxacin containing plate minus the diameter of the zone of inhibition in the water containing control plate.

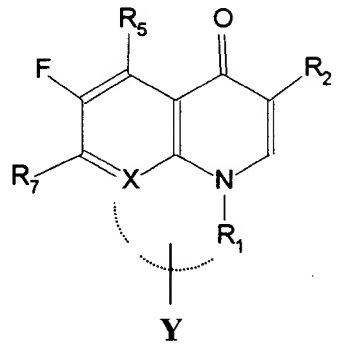
- # In the assay plates used, an agar well has a diameter of 7 mm; the minimum zone of inhibition observable is 8 mm in diameter. A zone less than 8 mm is considered as "nil".

The diameters of the zones of inhibition of all the test substances listed in the water-containing plate was nil, except for entry 4 which was 9 mm.

- ** R-isomer; * S-isomer; absence of an asterisk indicates that the substance is a racemic compound.

Table 4

Some Preferred Compounds of the Invention Displaying Inhibition of the mef Efflux Pump of *Streptococcus pneumoniae* 3514



Entry.	X	R_1	R_2	R_5	R_7	Difference in Zone diameter Z^1 (mm)
1.	C-OCH_3	c-C_3H_5	COOH	H	4-amino-3-methyl-piperidin-1-yl	26
2.	C-OCH_3	c-C_3H_5	$COOCH(CH_3)_2$	H	4-amino-3,3-dimethyl-piperidin-1-yl	11
3.	C-OCH_3	c-C_3H_5	$COO(CH_2)_3CH_3$	H	4-amino-3,3-dimethyl-piperidin-1-yl	11
4.	C-OCH_3	c-C_3H_5	$COOCH_2C_6H_5$	H	4-(t-butoxycarbonylamino)-3,3-dimethyl-piperidin-1-yl	13
5.	C-F	c-C_3H_5	COOH	NH_2	4-amino-3-methyl-piperidin-1-yl	20
6.	N	c-C_3H_5	COOH	H	(1∝,5∝,6∝)-3-N-benzylazabicyclo[3.1.0]hex-6-yl-amino	17
7.	N	C_6H_3 (2,4-F_2)	COOH	H	(1∝,5∝,6∝)-3-N-benzylazabicyclo[3.1.0]hex-6-yl-amino	15
8.	-C-OCH_2CH_2C*H(CH_3)		COOH	H	4-aminopiperidin-1-yl	13

[1]Z = the difference of the diameter of the zone of inhibition in the levofloxacin containing plate minus the diameter of the zone of inhibition in the water containing control plate.

** R-isomer; * S-isomer; absence of an asterisk indicates that the substance is a racemic compound.

Differences in An Efflux Pump Bearing Strain (*S. aureus* 1199B) to Efflux Different Fluoroquinolone Antibiotics

Bacterial strains : . *S.aureus* 1199 (NorA$^-$) and *S.aureus* 1199B (NorA$^+$) were kindly provided by Prof. G.W.Kaatz.

Antibiotics and reserpine solution preparation : The stock solution of fluoroquinolone antibiotcs and reserpine were prepared by dissolving test compounds in DMSO.

MIC Determination. Minimum Inhibitory Concentrations (MICs) were determined by NCCLS recommended agar dilution method[1] employing Mueller Hinton agar, Difco, Overnight grown culture of test strains *S.aureus* 1199 (Nor A$^-$) and *S. aureus* 1199B (Nor A$^+$) in Tryptic Soya Broth (TSB) were adjusted to deliver 10^4 cfu/spot using Denley's multipoint inoculator (Applied Quality Services, UK). Antibacterial agents were evaluated using 2 fold dilutions in the concentration range of 0.0125 – 50 mcg/ml in the absence and presence of (20mcg/ml) efflux pump inhibitor, reserpine. The plates were incubated at 35°C for 18 h. MICs were read as the lowest concentration of drug that completely inhibited bacterial growth.

[1] National Committee for Clinical Laboratory Standards. (2000). M7-A5. 5th edn. National Committee for Clinical Laboratory Standards, Wayne.PA

Table 1 MICs of fluoroquinolone antibiotics in a Nor A efflux pump bearing strain, *S. aureus* 1199B, in the absence of and presence of the efflux pump inhibitor, reserpine.

Antibiotics	MIC (mcg/ml)		Lowering of MIC (X times) In the presence of Reserpine
	S. aureus 1199B (Nor $A^{+)}$	*S. aureus* 1199B (Nor $A^{+)}$ + Reserpine (20 mcg/ml)	
Ciprofloxacin	6.25	0.8	8
Sitafloxacin	0.4	0.05	8
Norfloxacin	12.5	3.12	4
Clinafloxacin	0.2	0.05	4
Gemifloxacin	0.2	0.05	4
Levofloxacin	0.8	0.4	2
Gatifloxacin	0.4	0.2	2
Moxifloxacin	0.2	0.2	0

- The more the lowering of MIC , the higher the efflux of antibiotic in *S. aureus* 1199B

Table 2. Evidence of presence of resistance mechanism other than efflux in *S. aureus* 1199B (Nor A^{+})

Antibiotics	MIC (mcg/ml)			X times Higher MICs Of (N) compared to (L)
	S. aureus 1199 Nor A^{-} (L)	*S. aureus* 1199B (Nor $A^{+)}$ (M)	*S.aureus* 1199B (Nor $A^{+)}$ + Reserpine (20 mcg/ml) (N)	
Norfloxacin	3.12	12.5	3.12	0
Gemifloxacin	0.05	0.2	0.05	0
Ciprofloxacin	0.4	6.25	0.8	2
Sitafloxacin	0.025	0.4	0.05	2
Levofloxacin	0.2	0.8	0.4	2
Gatifloxacin	0.1	0.4	0.2	2
Clinafloxacin	0.0125	0.2	0.05	4
Moxifloxacin	0.05	0.2	0.2	4

- MICs of antibiotics in presence of reserpine for *S.aureus* 1199B ($NorA^{+}$) not matching with those of sensitive *S. aureus* 1199 ($NorA^{-}$) is indicative of other underlying resistance mechanisms of action in *S. aureus* 1199 ($NorA^{+}$) Strain.

F-563 Session 052

Clastogenicity and Human Topoisomerase II Inhibition Profiles of Non-Fluorinated Quinolones (NFQs)

V. A. MURPHY, B. LEDOUSSAL, P. J. RENICK, T. L. TWINEM, and T. W. MORRIS*

Procter & Gamble Pharmaceuticals, Mason, OH, USA

41st Interscience Conference on Antimicrobial Agents and Chemotherapy September 22-25, 2001 Chicago, Illinois

*Contact at: morris.tw@pg.com

Abstract

Background: Previously we reported a series of Non-Fluorinated Quinolones (NFQs) with potent antibacterial activity and sharply reduced genotoxicity relative to their 6-fluoroquinolone analogs (FQs). To test if additional NFQs also show reduced genotoxicity, an expanded set of 6 NFQ/FQ pairs (including clinafloxacin and gatifloxacin benchmarks) were profiled in vitro by micronucleus induction and human topoisomerase II inhibition assays. **Methods:** In vitro clastogenicity was assessed by measuring micronucleus induction in Chinese hamster ovary cells without metabolic activation over a concentration range of 1.6 – 2500 ug/mL. Inhibition of human topoisomerase II catalytic activity was measured using relaxation of supercoiled plasmid DNA over a concentration range of 1 – 600 ug/mL. **Results:** No micronucleus induction was observed for 3 of the NFQs, while micronucleus induction Lowest Observable Effect Level (LOEL) values for the remaining 3 NFQs ranged from 400 – 1000 ug/mL. For all 6 NFQ/FQ pairs, the corresponding FQ was at least 2.5 to >15.6-fold more potent for micronucleus induction. LOELs for the 6 corresponding FQs ranged from 64 – 400 ug/mL, including 160 and 400 ug/mL for clinafloxacin and gatifloxacin, respectively. In human topoisomerase II assays, IC50s for these NFQs were >600, >600, >600, 600, 600, and 200 ug/mL. In contrast, IC50s for the 6 corresponding FQs were 600, 400, 300, 400, 300, and 300 ug/mL, respectively. **Conclusion:** For these NFQ/FQ pairs, the 6-H quinolones clearly show reduced in vitro clastogenicity, while still retaining significant antibacterial potency. Although these NFQs are consistently less genotoxic in vitro than corresponding FQs, substitution at the 7 and 8 positions still influenced genotoxicity within this NFQ series according to previously established trends for FQs.

Introduction

Antibacterial quinolone drugs act by inhibiting the function of essential type II bacterial DNA topoisomerases [1]. However, many potent and broad spectrum quinolones (including clinically used agents) also induce genotoxicity when tested in mammalian cell model systems [2]. Although the precise molecular mechanism(s) remain elusive, the genotoxic effects of quinolone antibacterials are presumed to result from overlapping inhibition of similar type II topoisomerases present in mammalian cells. In previous reports [3,4,5], new 6-H substituted quinolones (NFQs) were described which show unique, unexpected biological properties relative to their 6-F substituted counterparts (FQs). Specifically, some of these novel NFQs display surprising antibacterial potency against problematic drug-resistant pathogens (e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*) while also demonstrating significantly reduced potential for mechanism-based genotoxicity. This unique combination of antibacterial potency and reduced genotoxicity thus challenged previously established SAR trends for antibacterial quinolones [2]. Here, we summarize key antibacterial, enzymatic, and genotoxic endpoints for 6 selected NFQ/FQ pairs which illustrate unique aspects of structure-activity-genotoxicity relationships for NFQ antibacterials.

Methods

Antibacterial Compounds and Assays: Test compounds were synthesized at P&G Pharmaceuticals. Growth inhibition MICs were measured against *S. aureus* MJ273 and *E.coli* ES142 by standard microbroth dilution assays [6].

Topoisomerase II Assays: Human topoisomerase II (p170 form) was purchased from Topogen, Inc. (Columbus, OH) and assayed for relaxation activity according to the manufacturer's instructions, with supercoiled pBR322 plasmid DNA as the substrate. Reaction products were separated on 1% agarose TAE gels, stained with ethidium bromide, and visually inspected to determine IC50 values.

Micronucleus Induction Assays: In vitro micronucleus screens were conducted at Covance Laboratories, Inc. (Vienna, VA). Chinese Hamster Ovary (CHO) cells in complete McCoy's 5A medium were exposed to test agents for 4 hours, then washed and reincubated for 16 hours in fresh media plus 3 ug/mL cytochalasin B. Cells were then fixed in methanol:acetic acid, stained with acridine orange, and binucleated cells were scored for micronuclei formation [7]. Lowest Observable Effect Levels (LOELs) were reported as the lowest non-cytotoxic compound concentrations resulting in ≥4.0% micronucleated cells and were significantly different ($p \leq 0.05$) relative to vehicle controls.

Table 1. In Vitro Activity Comparison of Fluorinated (FQs) and Non-Fluorinated Quinolones (NFQs)

NFQ/FQ Pair	Compound	R6	R7	R8	Micronucleus LOEL (ug/mL)	Topo II IC50 (ug/mL)	*S. aureus* MJ 273 MIC (ug/mL)	*E. coli* ES142 MIC (ug/mL)	Core Structure
Pair 1	PGE-5103714	H	3-amino-pyrrolidine	Cl	>400	600	0.06	0.03	
	Clinafloxacin	F		Cl	160	400	0.016	0.008	
Pair 2	PGE-9098633	H	3-amino-pyrrolidine	F	>400	200	0.12	0.03	
	PGE-1842612	F		F	64	300	0.016	0.004	
Pair 3	PGE-9098633	H	3-amino-pyrrolidine	OMe	1000	>600	≤0.06	≤0.06	
	PGE-79733121	F		OMe	64	400	0.03	0.016	
Pair 4	PGE-494276	H	3-methyl-piperazine	OMe	>2500	600	0.5	0.12	
	Gatifloxacin	F		OMe	400	300	0.06	0.06	
Pair 5	PGE-9509924	H	3-amino-piperidine	OMe	400	>600	0.016	0.03	
	PGE-7527720	F		OMe	160	300	0.06	0.12	
Pair 6	PGE-9262932	H	3-aminoethyl-pyrrolidine	OMe	1000	>600	≤0.008	0.03	
	PGE-9215875	F		OMe	400	600	0.008	0.008	

Fig. 1. Micronucleus Induction

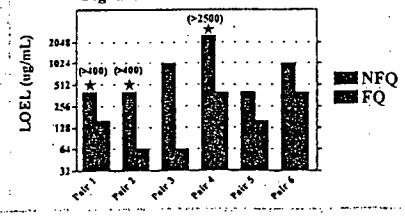
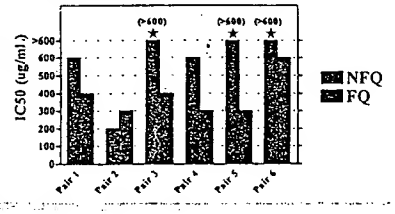


Fig. 2. Topoisomerase II Inhibition



Results & Conclusions

- 6 of 6 NFQs bearing various substitutions at both the R7 and R8 positions showed reduced micronucleus induction relative to their FQ counterparts. 5 of these 6 NFQs also showed reduced human topoisomerase II inhibition (Table 1, and Fig. 2).
- These data suggest that across these 6 pairs, NFQs have substantially reduced potential for in vivo genotoxicity.
- While the relative antibacterial potency of NFQs varied wide these 6 pairs (Table 1), selected NFQs (especially PGE-9509924 and PGE-9262932) were highly active, with MICs against representative *S. aureus* and *E. coli* that were comparable to their respective FQ counterparts.
- Although these NFQs are consistently less genotoxic in vitro than corresponding FQs (Fig. 1 and Fig. 2), substitutions at both the 7 and 8 positions within the NFQ series still influenced genotoxicity according to previously established trends for FQs (Table 1).

References

1. Hooper, D. C. 1999. Mode of action of fluoroquinolones. Drugs. 58; Suppl 2: 6-10.
2. Suto, M. J., J. M. Domagala, G. E. Roland, G. B. Mailloux, and M. A. Cohen. 1992. Fluoroquinolones: relationships between structural variations, mammalian cell cytotoxicity, and antimicrobial activity. J. Med. Chem. 35:4745-4750.
3. Ledoussal, B., J. K. Almstead, S. M. Flaim, C. P. Gallagher, J. L. Gray, X. E. Hu, N. K. Kim, H. D. McKeever, C. J. Miley, T. L. Twinem, and S. X. Zheng. 1999. Novel non-fluoroquinolones, structure-activity, and design of new, potent and safe agents. Abstract F-0544, 39th ICAAC, San Francisco, CA.
4. Roychoudhury S., T. L. Twinem, K. M. Makin, E. J. McIntosh, B. Ledoussal, and C. E. Catrenich. 2001. Activity of non-fluorinated quinolones (NFQs) against quinolone-resistant *Escherichia coli* and *Streptococcus pneumoniae*. J. Antimicrob. Chemother. 48:29-36.
5. Roychoudhury S, C. E. Catrenich, E. J. McIntosh, H. D. McKeever, K. M. Makin, P. M. Koenigs, and B. Ledoussal. 2001. Quinolone resistance in Staphylococci: activities of new nonfluorinated quinolones against molecular targets in whole cells and clinical isolates. Antimicrob. Agents Chemother. 45:1115-1120.
6. National Committee for Clinical Laboratory Standards. 1997. Method for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Doc. M7-A4.
7. Ciarrocchi, V., M. J. Suto, and J.C. Thiess. 1993. High Capacity in vitro micronucleus assay for assessment of chromosome damage. Mutat. Res. 291: 227-236.

52/F564

New 7-Piperidinyl Non-Fluorinated-Quinolones (NFQs), Synthesis and Structure-Activity Relationships

B. Ledoussal*, J.K. Almstead, C.P. Gallagher, J.L. Gray, X.E. Hu, N.K. Kim, H.D. Mckeever, C.J. Taylor, T.L. Twinem
Procter & Gamble Pharmaceuticals, Mason, OH

*Corresponding author:
[illegible]

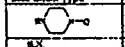
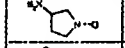
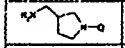
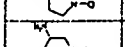
Abstract

Background: The early Fluoroquinolones have potent Gram negative coverage but typically are weaker on Gram positive organisms. During the numerous investigations aiming at improving 6-fluoroquinolones Gram positive activity, a strong parallel between the substitution favorable for Gram positive activity and worsening of genotoxicity was found. We previously reported(1) that substitution of the fluorine at the 6 position of the fluoroquinolones by a hydrogen represents a way to lower genotoxicity and that a judicious choice of the substitution at the 1, 8 and 7 positions of NFQs can provide a very potent Gram positive spectrum while maintaining a good Gram negative coverage. The preferred substituents at the 7 position were found to be of the 3-aminoazabicyclo-pyridinyl and the 3-amino-piperidinyl type. Results: In this communication, we will describe the synthesis of new 4 and 5 substituted 3-amino-piperidines and will show the influence of these new side chains on NFQs antibacterial activity. Our data show that substitutions at the 4 or 5 positions of the side chain can significantly impact antibacterial activity. 3-D comparisons with previously reported quinolones side chains of the pyrrolidine series will be presented. Conclusions: This work showed that 3-aminopiperidinyl side chains represent an avenue to generate series of multiple and diverse NFQs having potent broad spectrum and other interesting pharmacological properties.

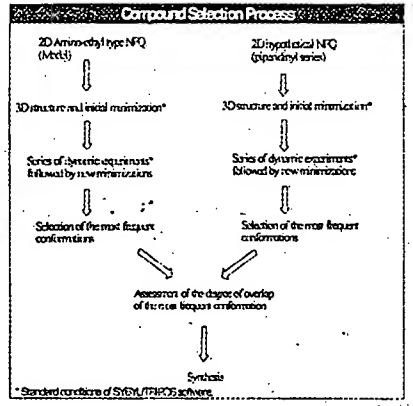
Background

The majority of the 7 position quinolone side chains can be arranged into three main categories: the piperazinyl, pyrrolidinyl and piperidinyl type side chains. Additionally, three sub-families of side chains were generated using the pyrrolidine backbone. The table below shows these families of compounds, their corresponding spectrum and some representatives of each family.

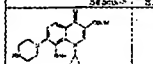
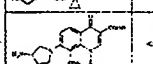
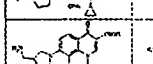
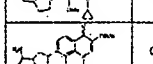
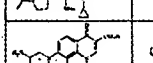
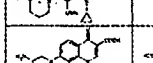
As previously reported (2), one of the most potent of the Gram-positive quinolones have a 3-amino-ethyl-pyrrolidine type side chain which represents a significant improvement versus their 3-amino-methyl-pyrrolidine homologs. Hypothesizing that this difference may be a consequence of an additional hydrophobic interaction, we attempted to reproduce a similar type of interactions in the piperidinyl series.

Side Chain Type	Gram Positive Spectrum	Gram Negative Spectrum	Examples
	+/-	++	- Ciprofloxacin - Levofloxacin
	+	+	- Tosufloxacin - Clinafloxacin
	+	+/-	
	++	+	- Prerrafloxacin
	+	+	- G-35

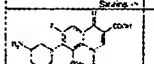
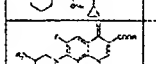
Compound Selection Process



MIC table (mg/l)

Structure	S.aureus	QR S.aureus	S.pneumoniae	E.coli	P.aeruginosa
	1	>32	8	0.5	4
	<=0.06	4	1	<=0.06	0.5
	<=0.06	1	0.12	0.12	2
	0.008	0.06	0.016	0.03	0.25
	0.016	0.25	0.03	0.03	0.5
	<=0.008	0.06	<=0.008	<=0.008	0.5

MIC Table Fluorinated Series (mg/l)

Structure	S.aureus	QR S.aureus	S.pneumoniae	E.coli	P.aeruginosa
	0.03	0.5	0.12	0.06	1
	0.008	0.125	0.03	0.016	1

Conclusions

- As previously disclosed, the contribution of the 7 position side chain has a more dramatic effect on antibacterial activity in the NFQ series than in the Fluoroquinolone series.
- The presence of an additional small lipophilic moiety in the vicinity of the terminal nitrogen attached to the 7 side chain improves significantly the overall antibacterial potency of the corresponding NFQ or Fluoroquinolone. In the pyrrolidine as well as piperidine series.
- Using small molecule modeling and a simple algorithm, a new type quinolone antibacterial side chain leading to potent broad spectrum agents has been designed.
- This work indicate that further investigation in the NFQ series can lead to new type of compounds having extremely promising antibacterial properties.

References

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Abstract: F-2142

Citation: Abstracts of the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, September and December 2001, page 251

Comparison of High-Field NMR, Circular Dichroism, and Gel Electrophoresis for Assessing Binding of Antibacterial Agents and Topoisomerase Inhibitors to DNA.

K.M. MAKIN, A.F. RUSSELL, T.L. TWINEM, C.E. CATRENICH, B. LEDOUSSAL, T.W. MORRIS

Procter & Gamble Pharmaceuticals, Mason, OH

Background: As part of an antibacterial drug discovery program to identify inhibitors of Type II bacterial topoisomerases (e.g. DNA gyrase), we sought practical methods for measuring binding of potential DNA topoisomerase inhibitors to double-stranded DNA. To assist rapid exclusion of topoisomerase inhibitors that act via nonselective binding to DNA substrates, we compared 3 experimental approaches for characterizing the binding of small molecule ligands to DNA. **Methods:** High-field solution NMR (based on linewidth broadening of aromatic protons from test ligands), circular dichroism (CD; based on DNA conformation shifts), and agarose gel electrophoresis (based on DNA mobility shifts) methods were compared against a common set of DNA binding ligands and DNA topoisomerase inhibitors. NMR experiments assessed binding to sonicated calf-thymus DNA, while CD and gel electrophoresis experiments assessed binding to supercoiled pBR322 bacterial plasmid DNA. **Results:** When challenged by known DNA binders (e.g. ethidium bromide, aminoacridine) and benchmark topoisomerase inhibitors (e.g. ciprofloxacin, novobiocin), the gel electrophoresis method gave inconsistent, non-quantitative gel shift profiles. The CD method was more predictive but was also labor-intensive and highly susceptible to minor method variations. The NMR method was clearly the most predictive, quantitative, and robust technique. **Conclusions:** The NMR method provides reliable and quantitative rank ordering of strong DNA binders (e.g. ethidium bromide), moderate binders (e.g. chloroquine), and weak DNA binders (e.g. ciprofloxacin). These data can be used to guide the rational design and optimization of antibacterials that selectively target Type II bacterial topoisomerases.

1: J Toxicol Environ Health. 1996 Feb 9;47(2):115-23.

Effects of new quinolone antibacterial agents on mammalian chromosomes.

Shimada H, Itoh S.

Developmental Research Laboratory, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

The clastogenic effects of several new quinolones (ciprofloxacin, enoxacin, levofloxacin, nalidixic acid, ofloxacin, pipemidic acid, and N1-cyclopropyl quinolones for drug candidate) were studied cytogenetically using Chinese hamster lung cells (CHL) and the mouse micronucleus test. Some N1 cyclopropyl quinolones strongly induced chromosomal aberration on CHL cells, and some, but not all, were also capable of inducing micronuclei in mouse bone marrow cells. Levofloxacin showed weak clastogenicity in CHL cells but did not induce either micronuclei in mouse bone marrow or unscheduled DNA synthesis (UDS) in rat hepatocytes when administered to intact live animals. The lack of concordance between in vitro and in vivo assays could reflect the differences in the tissue levels of the drugs and the in vitro conditions.

PMID: 8598568 [PubMed - indexed for MEDLINE]

1: Toxicol Lett. 1999 Jan 11;104(1-2):43-8.

Chromosomal aberrations in human lymphocytes exposed in vitro to enrofloxaci and ciprofloxacin.

Gorla N, Garcia Ovando H, Larripa I.

Facultad de Agronomia y Veterinaria, UNRC, Rio Cuarto, Argentina.

Chromosomal aberrations were evaluated in cultures of human peripheral lymphocytes from eight healthy donors, exposed to the antimicrobial enrofloxacin (EFX) or to its major metabolite ciprofloxacin (CFX). In both treatments cultures revealed an increase in the chromosomal aberratio level, detected as chromatid and chromosome breaks and gaps. Control cultures analysis revealed 3. +/- 0.6 chromosomal aberrations per 100 cells while treated cultures exhibited 8.3 +/- 0.8 and 9.6 +/ 1.2 aberrations at 5 and 50 microg/ml of EFX respectively. In CFX treated cultures it was found 5.6 +/- 1.3 and 7.7 +/- 3.5 aberrations/100 cells at 5 and 25 microg/ml antimicrobial concentration. The results suggested a genotoxic effect of EFX and CFX in the system used ($P < 0.001$). A reduction in the mitotic index and fuzzy metaphases were observed at 50 microg/ml of CFX, indicating a cytotoxic effect produced by this antimicrobial.

PMID: 10048748 [PubMed - indexed for MEDLINE]